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(54) Title: A DNA MOLECULE ENCODING A VARIANT α_{2B}-ADRENOCEPTOR PROTEIN, AND USES THEREOF

(57) Abstract: This invention relates to a DNA sequence comprising a nucleotide sequence encoding a variant α_{2B}-adrenoceptor protein and to said variant α_{2B}-adrenoceptor protein as well as a method for screening a subject to determine if said subject is a carrier of a variant gene that encodes said variant α_{2B}-adrenoceptor. Further this invention relates to a method for treating a mammal suffering from vascular contraction of coronary arteries, said method comprising the step of administering a selective α_{2B}-adrenoceptor antagonist to said mammal and to transgenic animals comprising a human DNA molecule encoding human α_{2B}-adrenoceptor or said variant α_{2B}-adrenoceptor.

A DNA MOLECULE ENCODING A VARIANT α_{2B}-ADRENOCEPTOR PROTEIN, AND USES THEREOF

FIELD OF THE INVENTION

This invention relates to a DNA molecule encoding a variant human α_{2B}-adrenoceptor, said variant α_{2B}-adrenoceptor protein and a method to assess the risk of individuals to suffer from vascular contraction of coronary arteries in mammals as well as a method for the treatment of vascular contraction of coronary arteries. This invention also relates to transgenic animals comprising a human DNA molecule encoding human α_{2B}-adrenoceptor or said variant α_{2B}-adrenoceptor.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference.

The α_2-adrenoceptors (α_2-ARs) mediate many of the physiological effects of the catecholamines norepinephrine and epinephrine. Three genetic subtypes of α_2-adrenoceptors are known in humans and other mammals, denoted as α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors. The human genes encoding the receptors are located on chromosomes 10, 2 and 4, respectively. No splice variants are known to exist of these receptors, as the genes are intronless. The tissue distributions and physiological and pharmacological functions of the receptor subtypes have been reviewed e.g. by MacDonald et al. (1997) and Docherty (1998). Based on recent studies with gene-targeted and transgenic mice, α_{2A}-adrenoceptors mediate most of

the pharmacological actions ascribed to currently available α_2-adrenoceptor agonists, including inhibition of neurotransmitter release, central hypotensive and bradycardic effects, sedation and anesthesia, and analgesia. The same studies indicate that α_{2B}-adrenoceptors mediate peripheral vasoconstriction in response to agonist activation (Link et al. 1996, Macmillan et al. 1996). Other physiological or pharmacological effects have not been associated with certainty with this receptor subtype. The α_{2C}-adrenoceptor subtype appears to be involved in regulation of complex behaviors. It is not known that this subtype would have important functions in peripheral tissues outside the central nervous system or in cardiovascular regulation.

Coronary heart disease (CHD), like many other common disorders, arises from complex interactions between genetic and environmental factors. It is reasonable to assume that functionally important genetic variation in mechanisms important for the regulation of vascular functions, including the coronary vasculature, will be found to be associated with the pathogenesis and therapy of CHD. A variant form of the human α_{2B}-AR gene was recently identified (Heinonen et al., 1999). The variant allele encodes a receptor protein with a deletion of three glutamate residues in an acidic stretch of 18 amino acids (of which 15 are glutamates) located in the third intracellular loop of the receptor polypeptide. This acidic stretch is a unique feature in the primary structure of α_{2B}-AR in comparison to α_{2A}-AR and α_{2C}-AR, suggesting that the motif has a distinct role in the function of α_{2B}-AR. Amino acid sequence alignment of α_{2B}-AR polypeptides of different mammals reveals that the acidic stretch is highly conserved among the α_{2B}-ARs of mammals and that the acidic stretch is long in humans in comparison to other species. This suggests that the motif is important for the functionality of the receptor, and that the short form (D for "deletion") probably represents the ancestral form and the long form (I for "insertion") could well represent a more recent allelic variant in humans. Jewell-Motz and Liggett (1995) studied the *in vitro* functions of this stretch using site-

directed mutagenesis to delete as well as to substitute 16 amino acids of the stretch. Their results suggest that this acidic motif is necessary for full agonist-promoted receptor phosphorylation and desensitization.

Based on the vasoconstrictive property of α_{2B}-AR in mice and the involvement of this acidic region in the desensitization mechanism of the receptor, we hypothesized that the deletion variant confers reduced receptor desensitization and therefore augmented vasoconstriction that could be associated with cardiovascular pathologies. To test this hypothesis, we carried out a 4-year prospective study in 912 middle-aged Finnish men.

OBJECT AND SUMMARY OF THE INVENTION

One object of this invention is to provide a DNA sequence of a variant human α_{2B}-adrenoceptor gene and the corresponding variant α_{2B}-adrenoceptor protein.

Another object of the invention is to provide a method for screening a subject to assess if an individual is at risk to suffer from vascular contraction of coronary arteries.

A third object of the invention is to provide a method for the treatment of vascular contraction of coronary arteries of mammals.

A fourth object of the invention is to provide a transgenic animal with a gene encoding a human α_{2B}-adrenoceptor or said variant thereof.

Thus, according to one aspect the invention concerns a DNA sequence comprising a nucleotide sequence encoding a variant α_{2B}-adrenoceptor protein with a deletion of at least 1 glutamate from a glutamic acid repeat element of 12 glutamates, amino

acids 298–309, in an acidic stretch of 18 amino acids 294–311, located in the 3rd intracellular loop of the receptor polypeptide.

The invention further concerns a variant α_{2B}-adrenoceptor protein with a deletion of at least 1 glutamate from a glutamic acid repeat element of 12 glutamates, amino acids 298–309, in an acidic stretch of 18 amino acids 294–311, located in the 3rd intracellular loop of the receptor polypeptide.

According to another aspect the invention concerns a method for screening a subject to determine if said subject is a carrier of a said variant gene with both alleles encoding a said variant α_{2B}-adrenoceptor, i.e. to determine if said subject's genotype of the human α_{2B}-adrenoceptor is of the deletion/deletion (D/D) type, comprising the steps of

a) providing a biological sample of the subject to be screened,

b) providing an assay for detecting in the biological sample the presence of

i) the insertion/insertion (I/I) or deletion/insertion (D/I) genotypes of the human α_{2B}-adrenoceptor, or

ii) the D/D genotype of the human α_{2B}-adrenoceptor, and

c) assessing at least one of the two following

i) an individual's risk to develop a disease involving vascular contraction of coronary arteries, or

ii) an individual's need for α_{2B}-selective or α_{2B}-nonselective α_2-adrenoceptor antagonist therapy,

based on whether said subject is of said D/D genotype or not.

According to a third aspect the present invention concerns a method for treating a mammal suffering from vascular contraction of coronary arteries, said method

comprising the step of administering a selective α_{2B}-adrenoceptor antagonist to said mammal.

According to a fourth aspect the present invention concerns a transgenic animal which carries a human DNA sequence comprising a nucleotide sequence encoding a human α_{2B}-adrenoceptor protein or a variant thereof.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a DNA molecule encoding a variant human α_{2B}-adrenoceptor, said variant α_{2B}-adrenoceptor protein and a method to assess the risk of individuals to suffer from vascular contraction of coronary arteries in mammals as well as a method for the treatment of vascular contraction of coronary arteries. The present invention also relates to transgenic animals comprising a human DNA molecule encoding a human α_{2B}-adrenoceptor or said variant α_{2B}-adrenoceptor protein.

The word treating shall also be understood to include preventing.

The concept "a deletion of at least 1 glutamate from a glutamic acid repeat element of 12 glutamates" refers to any deletion of 1 to 12 glutamates irrespective of the specific location in, or how many glutamates from said repeat element of 12 glutamates, amino acids 298–309 (SEQ ID NO: 4), in an acidic stretch of 18 amino acids 294–311 located in the 3rd intracellular loop of the receptor polypeptide are deleted.

The concept "deletion/deletion (D/D) genotype of the human α_{2B}-adrenoceptor", in short "D/D genotype", refers to a genotype of an individual having both α_{2B}-adrenoceptor alleles code for a variant α_{2B}-adrenoceptor with a deletion of at least 1 glutamate from a glutamic acid repeat element of 12 glutamates, amino

acids 298–309, in an acidic stretch of 18 amino acids 294–311 (SEQ ID NO: 4), located in the 3rd intracellular loop of the receptor polypeptide. Correspondingly "deletion/insertion (D/I) genotype" refers to a genotype having one of the gene alleles code for an α_{2B}-adrenoceptor with a said deletion and the other without a said deletion, i.e. with a respective insertion, and thus the "insertion/insertion (I/I) genotype" refers to a genotype having both alleles code for an α_{2B}-adrenoceptor without said deletion or deletions.

We recently identified a common variant form (SEQ ID NO: 1) of the human α_{2B}-AR gene (SEQ ID NO: 3). This variant gene encodes a receptor protein (SEQ ID NO: 2) with a deletion of 3 glutamates, amino acids 307-309, from a glutamic acid (Glu) repeat element of 12 glutamates, amino acids 298–309, in an acidic stretch of 18 amino acids 294–311 (SEQ ID NO: 4), located in the 3rd intracellular loop of the receptor polypeptide. This variant gene (SEQ ID NO: 1) was associated with decreased basal metabolic rate (BMR) in a group of obese Finnish subjects (Heinonen et al. 1999). Of the 166 obese subjects, 47 (28 %) were homozygous for the long 12 glutamate repeat element (Glu^{12}/Glu^{12}), whereas 90 (54 %) were heterozygous (Glu^{12}/Glu^{9}) and 29 (17 %) were homozygous for the short form (Glu^{9}/Glu^{9}).

The results to be presented below show that in a population-based cohort of 912 Finnish middle-aged men subjects homozygous for the short form (Glu^{9}/Glu^{9}) described above, thus representing a deletion/deletion (D/D) genotype of the α_{2B}-adrenoceptor, have a significantly elevated risk for acute coronary events in a four-year follow-up study. The risk for an acute coronary event, defined as definite or possible acute myocardial infarction (AMI) or prolonged (>20 min) chest pain requiring hospitalization, was increased 2.5 fold in subjects who had this D/D genotype. This increase in the risk for acute coronary events is as great as so far observed for any other genetic risk factor for acute coronary events or acute

myocardial infarction in a prospective population study. Also the frequency of a study subject having a history of coronary heart disease (CHD) as well as CHD in an exercise test was associated with this D/D genotype. Based on these results and previous publications referred to above it can be postulated that this D/D genotype is related to an impaired capacity to downregulate α_{2B}-adrenoceptor function during sustained receptor activation. Since altered α_{2B}-adrenoceptor function seems to be of relevance in the pathogenesis of a significant fraction of all cases of acute coronary events in subjects with this D/D genotype (homozygous Glu^9/Glu^9) we believe it could also be of relevance in subjects with the insertion/deletion (I/D) (heterozygous Glu^{12}/Glu^9) and insertion/insertion (I/I) (homozygous Glu^{12}/Glu^{12}) genotypes when other risk factors for AMI are present. Further, since this specific deletion of 3 glutamates, amino acids 307-309, from said glutamic acid repeat element of 12 glutamates, amino acids 298–309, in said acidic stretch of 18 amino acids 294–311, located in the 3rd intracellular loop of the receptor polypeptide seems to be of relevance in cases of AMI we believe that also other deletions, i.e. deletions of at least 1 glutamate, from said glutamic acid repeat element of 12 glutamates, amino acids 298–309, could be of relevance in the pathogenesis of AMI, because the 3rd intracellular loop of the receptor polypeptide it is located in seems to have an essential role in the downregulation of the α_{2B}-adrenoceptor.

Thus based on the results to be presented below and the publications referred to above an α_{2B}-adrenoceptor antagonist would be useful for treating a mammal suffering from vascular contraction of coronary arteries.

Furthermore, an α_{2B}-adrenoceptor antagonist selective for the α_{2B}-adrenoceptor subtype would be therapeutically beneficial for the treatment of a disease involving said vascular contraction of coronary arteries. Such a disease could be clinically expressed as chronic angina pectoris, specifically e.g. AMI, unstable angina pectoris or Prinzmetal's variant form of angina pectoris. If α_{2B}-adrenoceptor dependent

vasoconstriction is a causative factor in some cases of AMI, then antagonism of these receptors should restore coronary circulation and reduce the ischemic myocardial damage. An α_{2B}-adrenoceptor antagonist will relieve the vaso-constrictive component in the sustained ischemic episode of unstable angina pectoris, thus alleviating the symptoms and preventing AMI. Vasoconstriction is a key factor in the pathogenesis of Prinzmetal's angina, and an α_{2B}-adrenoceptor antagonist may resolve and prevent attacks. An α_{2B}-adrenoceptor antagonist will help to alleviate the vasoconstrictive component in all types of CHD, providing both symptomatic relief and protection from AMI.

α_{2B}-adrenoceptors mediate vascular contraction of coronary arteries, and genetic polymorphism present in the α_{2B}-adrenoceptor gene renders some subjects more susceptible to α_{2B}-adrenoceptor mediated vasoconstriction of coronary arteries and associated clinical disorders. These subjects will especially benefit from treatment with an α_{2B}-adrenoceptor antagonist, and will be at increased risk for adverse effects if subtype-nonselective α_2-agonists are administered to them. Therefore, a gene test recognizing subjects with a deletion variant of the α_{2B}-adrenoceptor gene will be useful in diagnostics and patient selection for specific therapeutic procedures. A gene test recognizing the D/D genotype of the α_{2B}-adrenoceptor is useful in assessing an individual's risk to develop AMI and other clinical disorders involving vascular contraction of coronary arteries related to the D/D genotype. A gene test recognizing the D/D genotype of the α_{2B}-adrenoceptor is useful in selecting drug therapy for patients with diseases involving vascular contraction of coronary arteries associated with the D/D genotype; subjects with the D/D genotype will especially benefit from therapy with α_2-adrenoceptor antagonists (α_{2B}-selective or nonselective). A gene test recognizing the D/D genotype of the α_{2B}-adrenoceptor is useful in selecting drug therapy for patients who might be at increased risk for adverse effects of α_2-adrenergic agonists; either, it will be possible to avoid the use

of α_2-agonists in such patients, or it will be possible to include a specific α_{2B}-antagonist in their therapeutic regimen.

The DNA sequence can be used for screening a subject to determine if said subject is a carrier of a variant gene. The determination can be carried out either as a DNA analysis according to well known methods, which include direct DNA sequencing of the normal and variant gene, allele specific amplification using the polymerase chain reaction (PCR) enabling detection of either normal or variant sequence, or by indirect detection of the normal or variant gene by various molecular biology methods including e.g. PCR-single stranded conformation polymorphism (SSCP) method or denaturing gradient gel electrophoresis (DGGE). Determination of the normal or variant gene can also be done by using a restriction fragment length polymorphism (RFLP) method, which is particularly suitable for genotyping large numbers of samples. Similarly, a test based on gene chip technology can be easily developed in analogy with many currently existing such tests for single-nucleotide polymorphisms. Thus such an test could be an assay carried out using a gene chip, microarray, strip, panel or similar combination of more than one genes, mutations or RNA expressions to be assayed.

The determination can also be carried out at the level of RNA by analyzing RNA expressed at tissue level using various methods. Allele specific probes can be designed for hybridization. Hybridization can be done e.g. using Northern blot, RNase protection assay or in situ hybridization methods. RNA derived from the normal or variant gene can also be analyzed by converting tissue RNA first to cDNA and thereafter amplifying cDNA by an allele specific PCR method.

As examples of useful α_{2B}-adrenoceptor antagonists can be mentioned imiloxan [2-(1-ethyl-2-imidazoyl)methyl-1,4-benzodioxan, ARC-239 [2-[2-(4-(2-methoxy-phenyl)piperazin-1-yl)ethyl]-4,4-dimethyl-1,3-(2H,4H)-isoquinolindione], prazosin

[1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl)piperazine] and chlorpromazine [2-chloro-N,N-dimethyl-10H-phenothiazine-10-propanamine].

The required dosage of the compounds will vary with the particular condition being treated, the severity of the condition, the duration of the treatment, the administration route and the specific compound being employed. A typical therapeutically effective daily dose administered, e.g. orally or by infusion, can vary from e.g. 0.1 μg to 10 mg per kilogram body weight of an adult person.

Influence of the variant gene sequence can be investigated in transgenic animals. A transgenic animal can be generated e.g. using targeted homologous recombination methodology. This will provide an ideal preclinical model to investigate and screen new drug molecules, which are designed to modify the influence of the variant gene.

The invention will be described in more detail in the experimental section.

EXPERIMENTAL SECTION

Determination of genomic alleles encoding the α_{2B}-adrenoceptor

PCR-SSCA analysis

The polymerase chain reaction-single stranded conformational analysis (PCR-SSCA) used to identify the genomic alleles encoding the α_{2B}-adrenoceptor was carried out as follows: The genomic DNA encoding the α_{2B}-adrenergic receptor was amplified in two parts specific for the intronless α_{2B}-adrenoceptor gene sequence (Lomasney et al. 1990). The PCR primer pairs for PCR amplification were as follows: Pair 1: 5′-GGGGCGACGCTCTTGTCTA-3′ (SEQ ID NO: 5) and 5′-GGTCTCCCCCTCCTCCTTC-3′ (SEQ ID NO: 6) (product size 878 bp),

pair 2: 5´-GCAGCAACCGCAGAGGTC-3´ (SEQ ID NO: 7) and 5´-GGGCAA-GAAGCAGGGTGAC-3´ (SEQ ID NO: 8) (product size 814 bp). The primers were delivered by KeboLab (Helsinki, Finland). PCR amplification was conducted in a 5 µl volume containing 100 ng genomic DNA (isolated from whole blood), 2.5 mmol/l of each primer, 1.0 mmol/l deoxy-NTPs, 30 nmol/l ^{33}P-dCTP and 0.25 U AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). PCR conditions were optimized using the PCR Optimizer™ kit (Invitrogen, San Diego, CA). Samples were amplified with a GeneAmp PCR System 9600 (Perkin Elmer Cetus). PCR products were digested with restriction enzymes for SSCA analysis. The product of primer pair 1 was digested with Dde I and Dra III (Promega Corp., Madison, WI). The product of primer pair 2 was digested with Alu I and Hinc II (Promega Corp.). The digested samples were mixed with SSCA buffer containing 95 % formamide, 10 mmol/l NaOH, 0.05 % xylene cyanol and 0.05 % bromophenol blue (total volume 25 µl). Before loading, the samples were denatured for 5 min at 95 °C and kept 5 min on ice. Three microliters of each sample were loaded on MDE™ high-resolution gel (FMC, BioProducts, Rockland, MA). The gel electrophoresis was performed twice, at two different running conditions: 6 % MDE gel at +4 °C and 3 % MDE gel at room temperature, both at 4 W constant power for 16 h. The gels were dried and autoradiography was performed by apposing to Kodak BioMax MR film for 24 h at room temperature.

Sequencing and genotyping

DNA samples migrating at different rates in SSCA were sequenced with the Thermo Sequenase™ Cycle Sequencing Kit (Amersham Life Science, Cleveland, OH).

For genotyping the identified 3-glutamic acid deletion, DNA was extracted from peripheral blood using standard methods. The α_{2B}-AR I/D genotype was determined by separating PCR-amplified DNA fragments with electrophoresis. Based on the

nature of the I/D variant, identification of the long and short alleles was achieved by their different electrophoretic migration rates due to their 9 bp size difference.

The region of interest was amplified using a sense primer 5'-AGGGTGTTTGTG-GGGCATCT-3' (SEQ ID NO: 9) and an anti-sense primer 5'-CAAGCTGAGGCC-GGAGACACT-3' (SEQ ID NO: 10) (Oligold, Eurogentec, Belgium), yielding a product size of 112 bp for the long allele (I) and 103 bp for the short allele (D). PCR amplification was conducted in a 10 µL volume containing ~100 ng genomic DNA, 1x buffer G (Invitrogen, San Diego, CA, USA), 0.8 mM dNTPs, 0.3 µM of each primer and 0.25 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, USA). Samples were amplified with a GeneAmp PCR System 9600 (Perkin Elmer Cetus). After initial denaturation at 94 °C for 2 minutes, the samples were amplified over 35 cycles. PCR amplification conditions were 96 °C (40 s), 69 °C (30 s) and 72 °C (30 s) followed by final extension at 72 °C for 6 minutes. The PCR products representing the long and short alleles were identified by two alternative methods.

1) The amplified samples were mixed with 4 µl of stop solution (Thermo Sequenase™ Cycle Sequencing kit), heated to 95 °C for 2 min, and loaded hot onto sequencing gels (Long Ranger™, FMC). The gels were dried and autoradiography was performed as previously described.

2) Separation of the amplified PCR products was performed with electrophoresis on a high-resolution 4 % Metaphor agarose gel (FMC Bioproducts, Rockland, Maine) and the bands were visualized by ethidium bromide staining. In both methods, the long (Glu^{12}) and short (Glu^{9}) alleles were identified based on their different electrophoretic migration rates.

Follow-up study

The above referred four-year follow-up study of 912 Finnish middle-aged men subjects including 192 subjects with a specific deletion/deletion (D/D) genotype of the α_{2B}-adrenoceptor is described in more detail in the following:

Knowing the vasoconstrictive property of α_{2B}-AR in mice and the possible involvement of the investigated acidic region in the desensitization mechanism of the receptor we hypothesized that the observed insertion/deletion allelic variation could be associated with cardiovascular pathologies such as AMI. To test this hypothesis, we carried out a four-year follow-up study in 912 middle-aged Finnish men with no prior history of AMI. The study was carried out as part of the Kuopio Ischemic Heart Disease Risk Factor Study (KIHD), which is an ongoing population-based study designed to investigate risk factors for cardiovascular diseases and related outcomes in men from eastern Finland (Salonen 1988). This area is known for its homogenous population (Sajantila et al. 1996) and high coronary morbidity and mortality rates (Keys 1980).

Of the 912 subjects, 192 (21 %) had the D/D genotype, 256 (28 %) had the I/I genotype and 464 (51 %) were heterozygous i.e. I/D. This genotype distribution is in Hardy-Weinberg equilibrium ($p = 0.46$).

Of the 37 cases that had an acute coronary event during the follow-up, 18 were classified as definite AMI, 12 as possible AMI and seven as prolonged chest pain. Among the subjects with the D/D genotype, 15 (8 %) had an acute coronary event during the follow-up time. The corresponding incidences for the I/I and the heterozygous genotypes i.e. I/D were 10 (4 %) and 12 (3 %). The observed cumulative incidence of acute coronary events differed significantly among the different genotypes ($p = 0.008$). No significant difference in the cumulative incidence of acute coronary events was found between the I/D and the I/I genotypes

(p = 0.4) (table 1). There was a significant difference (log-rank p = 0.0045) between the D/D subgroup and the other two genotypes combined in the cumulative event-free time in the Kaplan-Meier survival function, demonstrating that there is a consistently increased incidence of acute coronary events in the D/D subgroup.

The D/D genotype was associated with a 2.5 fold increased risk for an acute coronary event (95% CI = 1.3–4.8, p = 0.006) in comparison to the other two genotypes combined. The relative risk remained above 2 after adjustment for major CHD risk factors (table 2).

The D/D subgroup was not significantly different from the I/D + I/I subgroup in terms of many known major risk factors for CHD. From 87 variables in the study database only 5 were significantly different between the D/D and the I/D + I/I genotype subgroups: 1. there were more acute coronary events in the D/D subgroup (8 % vs. 3 %, p = 0.006), 2. history of CHD was more prevalent in the D/D subgroup (37 % vs. 29 %, p = 0.043), 3. the prevalence of CHD in exercise test was higher in the D/D subgroup (30 % vs. 22 %, p = 0.036), 4. mean hemoglobin level was higher in the D/D subgroup (149.0 g/l vs. 146.8 g/l, p = 0.005) and 5. mean dietary cholesterol intake (4-days) was lower in the D/D subgroup (411.6 mg vs. 440.1 mg, p = 0.033) (table 3). The first four observed differences support our hypothesis that the D/D genotype confers reduced receptor desensitization and therefore augmented vasoconstriction. This augmented vasoconstriction is the reason for the increased incidence of acute coronary events, the higher prevalence of CHD in exercise and history of CHD. We hypothesize that the increased level of hemoglobin is due to relative anoxia of tissues because of this augmented vasoconstriction.

To examine the possibility that the D/D genotype is a genetic marker for acute coronary events rather than a causative factor, we have searched the literature for known genetic risk factors for acute coronary events and AMI and their

chromosomal localization. All but one (Apo-B) are on different chromosomes than the α_{2B}-AR gene (chromosome 2) and the gene for Apo-B is neither in the physical nor the genetic vicinity of the α_{2B}-AR gene. Cox regression analysis revealed that the increased RR for acute coronary events in the D/D subgroup is not affected by the serum Apo-B concentration.

Taken together, the known biological properties of the α_{2B}-AR, the homogeneity of the Finnish population with its relatively high incidence of CHD, the study design, the relatively large representative study population and the clustering of the findings around one trait suggest that the D/D receptor allele is a causal genetic risk factor for acute coronary events.

Table 1: The cumulative incidence of acute coronary events among men with different genotypes of the α_{2B}-AR (p values are stated below)

Genotype		Events (% of men at risk)	Men at risk (% of all)
D/D	observed	15 (8)	192 (21)
	expected	7.8	
I/D	observed	12 (3)	464 (51)
	expected	18.8	
I/I	observed	10 (4)	256 (28)
	expected	10.4	
I/D + I/I	observed	22 (3)	720 (79)
	expected	29.2	
Total	observed	37 (4)	912 (100)

P values for the above table:

D/D vs. I/D vs. I/I	p = 0.008
D/D vs. I/D	p = 0.002
D/D vs. I/I	p = 0.038
I/D vs. I/I	p = 0.389
D/D vs. I/D + I/I	p = 0.005

Table 2: Relative risk (RR) and its 95% confidence interval (CI) for an acute coronary event – a comparison of each of the genotypes with the other two combined. Results of a Cox regression model for 37 acute coronary events in a population sample of 912 subjects

Genotype	Events/men at risk	RR (95% CI)	Adjusted RR (95% CI)
		p	p
D/D	15/192	2.5 (1.3–4.8)	2.3 (1.2–4.5)
		0.006	0.014
I/D	12/464	0.44 (0.2–0.9)	0.5 (0.2–1.0)
		0.020	0.052
I/I	10/256	1.03 (0.5–2.1)	0.96 (0.5–2.0)
		0.940	0.901

Adjustment was done for age, CHD in the family, high cholesterol in the family, hypertension and smoking

Table 3: List of all significant differences ($p<0.05$) between the D/D and the I/D+I/I genotype subgroups among 87 variables in the study database

Variable	D/D	I/D + I/I	p
Acute coronary events [event/n (%)]	15/192 (8)	22/720 (3)	0.006
Ischemic findings in exercise test [case/n (%)]	57/192 (30)	160/720 (22)	0.036
History of CHD [case/n (%)]	71/192 (37)	209/720 (29)	0.043
Mean blood haemoglobin [g/L]	149.0	146.8	0.005
Mean 4 day dietary cholesterol intake [mg]	411.6	440.1	0.033

% = Percent of men at risk

It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the specialist in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

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CLAIMS

1. A DNA sequence comprising a nucleotide sequence encoding a variant α_{2B}-adrenoceptor protein with a deletion of at least 1 glutamate from a glutamic acid repeat element of 12 glutamates, amino acids 298–309, in an acidic stretch of 18 amino acids 294–311, located in the 3rd intracellular loop of the receptor polypeptide.

2. The DNA sequence according to claim 1 comprising a nucleotide sequence encoding a variant α_{2B}-adrenoceptor protein with a deletion of 3 glutamates, amino acids 307–309, from said glutamic acid repeat element of 12 glutamates, amino acids 298–309, in said acidic stretch of 18 amino acids 294–311, located in the 3rd intracellular loop of the receptor polypeptide.

3. The DNA sequence according to claim 2 comprising the genomic nucleotide sequence of SEQ ID NO: 1.

4. The DNA sequence according to claim 1 wherein said DNA sequence is cDNA.

5. An RNA sequence comprising an RNA sequence corresponding to the DNA sequence of claim 1.

6. A variant α_{2B}-adrenoceptor protein having a deletion of at least 1 glutamate from said glutamic acid repeat element of 12 glutamates, amino acids 298–309, in said acidic stretch of 18 amino acids 294–311 , located in the 3rd intracellular loop of the receptor polypeptide.

7. A variant α_{2B}-adrenoceptor protein according to claim 6 having a deletion of 3 glutamates, amino acids 307–309, from said glutamic acid repeat element of

12 glutamates, amino acids 298–309, in said acidic stretch of 18 amino acids 294–311, located in the 3rd intracellular loop of the receptor polypeptide.

8. The variant protein according to claim 7 comprising the amino acid sequence of SEQ ID NO: 2.

9. An assay for determining the presence or absence of a variant gene as defined in claim 1.

10. An assay according to claim 9 wherein said assay is carried out using a gene chip, microarray, strip, panel or similar combination of more than one genes, mutations or RNA expressions to be assayed.

11. The assay according to claim 9 wherein the assay is a DNA-assay.

12. A method for determining the presence or absence in a biological sample of a DNA sequence as defined in claim 1, wherein said DNA, which appears in single stranded form (target nucleic acid), is brought into contact with a capturing nucleic acid probe and a detector nucleic acid probe, after which the complex "capturing probe-target nucleic acid-detector probe" is detected.

13. The method according to claim 12, wherein the capturing nucleic acid probe is attached or capable of attaching to a solid phase, and comprises the cDNA sequence according to claim 4, wherein a detected signal from the solid phase is an indication of the presence in the sample of a DNA as defined in claim 1.

14. The method according to claim 12, wherein the capturing nucleic acid probe is attached or capable of attaching to a solid phase, and comprises the cDNA corresponding to the gene coding an α_{2B}-adrenoceptor without the deletion defined in claim 1, wherein a detected signal from the solid phase is an indication of the absence in the sample of a DNA as defined in claim 1.

15. A method for screening a subject to determine if said subject is a carrier of a said variant gene with both alleles encoding a said variant α_{2B}-adrenoceptor , i.e. to determine if said subject's genotype of the human α_{2B}-adrenoceptor is of the deletion/deletion (D/D) type, comprising the steps of

a) providing a biological sample of the subject to be screened,

b) providing an assay for detecting in the biological sample the presence of

i) the insertion/insertion (I/I) or deletion/insertion (D/I) genotypes of the human α_{2B}-adrenoceptor, or

ii) the D/D genotype of the human α_{2B}-adrenoceptor, and

c) assessing at least one of the two following

i) an individual's risk to develop a disease involving vascular contraction of coronary arteries, or

ii) an individual's need for α_{2B}-selective or α_{2B}-nonselective α_2-adrenoceptor antagonist therapy,

based on whether said subject is of said D/D genotype or not.

16. The method according to claim 15 wherein the assay is a DNA-assay.

17. A capturing probe which comprises a single strand of the cDNA according to claim 4.

18. A capturing probe which comprises a single strand of the cDNA corresponding to the α_{2B}-adrenoceptor without the deletion defined in claim 1

19. A method for treating a mammal suffering from vascular contraction of coronary arteries, said method comprising administering a selective α_{2B}-adrenoceptor antagonist to said mammal.

20. The method according to claim 19 wherein said mammal suffers from a disease involving said vascular contraction of coronary arteries.

21. The method according to claim 20 wherein said disease is clinically expressed as coronary heart disease or chronic angina pectoris.

22. The method according to claim 20 wherein said disease is clinically expressed as acute myocardial infarction.

23. The method according to claim 21 wherein said chronic angina pectoris is unstable.

24. The method according to claim 21 wherein said chronic angina pectoris is clinically expressed as Prinzmetal's variant form.

25. A transgenic animal which carries a human DNA sequence comprising a nucleotide sequence encoding a variant α_{2B}-adrenoceptor protein with a deletion of at least 1 glutamate from a glutamic acid repeat element of 12 glutamates, amino acids 298–309, in an acidic stretch of 18 amino acids 294–311, located in the 3rd intracellular loop of the receptor polypeptide.

26. A transgenic animal according to claim 25 encoding a variant α_{2B}-adrenoceptor protein with a deletion of 3 glutamates, amino acids 307–309.

27. A transgenic animal which carries a human DNA sequence encoding a α_{2B}-adrenoceptor protein without said deletion of at least 1 glutamate from a glutamic acid repeat element of 12 glutamates, amino acids 298–309, in an acidic stretch of 18 amino acids 294–311, located in the 3rd intracellular loop of the receptor polypeptide.

SEQUENCE LISTING

<110> Snapir, Amir
Heinonen, Paula
Alhopuro, Pia
Karvonen, Matti
Koulu, Markku
Pesonen, Ullamari
Scheinin, Mika
Salonen, Jukka T
Tuomainen, Tomi-Pekka
Lakka, Timo A
Nyyssönen, Kristiina
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gcc gtg agc cgc gcg ctg gag tac aac tcc aag cgc acc ccg cgc cgc   384
Ala Val Ser Arg Ala Leu Glu Tyr Asn Ser Lys Arg Thr Pro Arg Arg
        115                 120                 125

atc aag tgc atc atc ctc act gtg tgg ctc atc gcc gcc gtc atc tcg   432
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ctg cgc atc tac ctg atc gcc aaa cgc agc aac cgc aga ggt ccc agg   624
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        275                 280                 285

tgt ggg gca tct cca gag gat gaa gct gaa gag gag gaa gag gag gag   912
Cys Gly Ala Ser Pro Glu Asp Glu Ala Glu Glu Glu Glu Glu Glu Glu
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            20                  25                  30

gtc atc ctg gct gtg ttg acc agc cgc tcg ctg cgc gcc cct cag aac  144
Val Ile Leu Ala Val Leu Thr Ser Arg Ser Leu Arg Ala Pro Gln Asn
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ctg ttc ctg gtg tcg ctg gcc gcc gcc gac atc ctg gtg gcc acg ctc  192
Leu Phe Leu Val Ser Leu Ala Ala Ala Asp Ile Leu Val Ala Thr Leu
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cgg cgc acg tgg tgc gag gtg tac ctg gcg ctc gac gtg ctc ttc tgc  288
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Thr Ser Ser Ile Val His Leu Cys Ala Ile Ser Leu Asp Arg Tyr Trp
            100                 105                 110

gcc gtg agc cgc gcg ctg gag tac aac tcc aag cgc acc ccg cgc cgc  384
Ala Val Ser Arg Ala Leu Glu Tyr Asn Ser Lys Arg Thr Pro Arg Arg
        115                 120                 125

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Ile Lys Cys Ile Ile Leu Thr Val Trp Leu Ile Ala Ala Val Ile Ser
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Leu Pro Pro Leu Ile Tyr Lys Gly Asp Gln Gly Pro Gln Pro Arg Gly
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cgc ccc cag tgc aag ctc aac cag gag gcc tgg tac atc ctg gcc tcc  528
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Ser Ile Gly Ser Phe Phe Ala Pro Cys Leu Ile Met Ile Leu Val Tyr
            180                 185                 190

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Leu Arg Ile Tyr Leu Ile Ala Lys Arg Ser Asn Arg Arg Gly Pro Arg
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His Gly Gly Ala Leu Ala Ser Ala Lys Leu Pro Ala Leu Ala Ser Val
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Cys Gly Ala Ser Pro Glu Asp Glu Ala Glu Glu Glu Glu Glu Glu Glu
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Leu Arg Ile Tyr Leu Ile Ala Lys Arg Ser Asn Arg Arg Gly Pro Arg
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Ala Lys Gly Gly Pro Gly Gln Gly Glu Ser Lys Gln Pro Arg Pro Asp
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His Gly Gly Ala Leu Ala Ser Ala Lys Leu Pro Ala Leu Ala Ser Val
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<223> Description of Artificial Sequence: PCR primer pair

<400> 9
agggtgtttg tggggcatct 20

<210> 10
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR primer pair

<400> 10
caagctgagg ccggagacac t 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 00/00913

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07K 14/47, C07K 14/72
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	The Journal of Clinical Endocrinology & Metabolism, Volume 84, No 7, 1999, PAULA HEINONEN et al, "Identification of a Three-Amino Acid Deletion in the .... Adrenergic Receptor That Is Associated with Reduced Basal Metabolic Rate in Obese Subjects", page 2430, column 2, line 33 - line 39, figures 1,2, page 2429 - 2433	1-18
Y	--	19-27
X	US 5595880 A (RICHARD L. WEINSHANK ET AL), 21 January 1997 (21.01.97), column 2, line 46 - column 3, line 19, figure 2	18
A	--	1-17,19-24

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 February 2001	01 -03- 2001
Name and mailing address of the ISA: Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Frida Plym Forshell/mj Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 00/00913

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J Am Coll Cardiol, Volume 33, No 6, 1999, Julius BK, "Alpha-adrenoceptor blockade prevents exercise-induced vasoconstriction of stenotic coronary arteries" page 1499 - page 1505 -- US 5861309 A (JONATHAN A. BARD ET AL), 19 March 1999 (19.03.99), column 4, line 35 - line 54 -- --------	19-24 25-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI00/00913

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see extra sheet

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Claims 15 and 16 relate to a diagnostic method practised on the human or animal body, and claims 19 -24 relate to a method for treatment of the human or animal body by therapy. Thus, the International Search Authority is not required to carry out an international search for these claims (Rule 39.1(iv)). Nevertheless, a search has been executed for claims 15,16 and 19-24.

INTERNATIONAL SEARCH REPORT

Information on patent family members

05/02/01

International application No.
PCT/FI 00/00913

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5595880 A	21/01/97	US 5053337 A	01/10/91
US 5861309 A	19/03/99	AU 677968 B	15/05/97
		AU 5165693 A	26/04/94
		DE 663014 T	10/10/96
		EP 0663014 A	19/07/95
		GR 95300067 T	31/01/96
		JP 8505044 T	04/06/96
		AU 718197 B	06/04/00
		AU 3420797 A	29/01/98
		CA 2145182 A	14/04/94
		EP 1063291 A	27/12/00
		EP 1063292 A	27/12/00
		ES 2085247 T	01/06/96
		US 5556753 A	17/09/96
		US 5714381 A	03/02/98
		US 6083705 A	04/07/00
		US 6156518 A	05/12/00
		WO 9408040 A	14/04/94